Development and characterization of processed, high-lipid piglet formula enriched with phospholipids and evaluation of its protein digestibility post-heat treatment

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1. ABSTRACT

Homogenization and heat-treatment of piglet formula could providers researchers who artificially rear neonatal pigs an efficiency, less added variability, and more flexibility in their studies. But the processing of piglet formula must be validated, as homogenization and heat-treatment both affect the surface load of the milk fat globule membrane(MFGM), a natural emulsifying agent in dairy products. It is hypothesized that heat-treatment of a high-lipid (8% w/v), homogenized piglet formula will have reduced digestibility in the piglet due to accumulated adsorption of whey protein on the MFGM surface that limits protease access to cleavage sites. Piglet formula was developed to mimic the nutrient profile of sow milk, processed with HTST and UHT conditions, and digested in-vitro by porcine pepsin. Aliquots were taken at 3, 5, 10, 15, 20, 30, 60, and 120 minutes and analyzed with SDS-PAGE using stain-free gels. The digestion of casein and whey proteins was evaluated by the change in their band intensities over time. Casein was largely digested within the first three minutes, while whey proteins were digested gradually. Overall digestion of protein was evaluated by the change in band intensity of the peptide fraction over digestion time. However, inadequate formula hydration rendered the results inconclusive, and more trials are required. Nonetheless, it was seen that UHT processing increased the viscosity of the piglet formula (UHT: 10.8±0.26, HTST: 4.9±0.26, control: 4.8±0.22 cP), which is important for stability and palatability considerations.

2. INTRODUCTION

Infant pigs serve as excellent models for the infant human due to the physiological and anatomical similarity of their digestive tracts and are thus commonly used by researchers to study nutrient absorption, and metabolism (Pond 1986). In artificially rearing these baby pigs, researchers must pay close attention to the piglets' macronutrient and micronutrient

requirements, as pigs grow rapidly—upwards of a 1000% body weight increase in 6 weeks (Altman and Dittmer 1962). Protein malnutrition of the young pig leading to kwashiorkor has been studied by Pond et al (1971), and others have investigated the death of baby pigs fed reconstituted whole milk that was severely heated prior to spray-drying; indigestion and scouring was observed in the deceased baby pigs (Braude et al 1970). Since this study, little work has been done to assess the effect of processing on digestibility of protein in formula for the baby pig, which must have a high lipid content. Processing here refers to both homogenization and subsequent heat-treatment.

Recently, researchers have paid attention to the MFGM and its protein-protein and protein-lipid interactions with whey and casein proteins throughout processing. During homogenization when the fat globules are reduced in size, the newly exposed surface area is stabilized by micellar and sub-micellar caseins that adsorb onto the MFGM preferentially over whey proteins (Lee and Sherbon 2002). During subsequent heat-treatment, heat-induced binding of whey proteins β-lactoglobulin and α-lactalbumin to the MFGM occurs (Corredig and Dalgleish 1996). Casein was not shown to interact with the MFGM upon homogenization and heat-processing (Lee and Sherbon 2002). It is our hypothesis that deposition of whey proteins on the MFGM during heat-processing of a high lipid (8% w/v), homogenized formula for the baby pig will result in reduced protein digestibility due to deposited proteins impeding digestive enzyme activity at its cleavage sites.

Changes to the surface proteins of the MFGM due to homogenization and heat treatment may impact stability of the formula, as the composition of the MFGM, a natural emulsifying agent, contributes to the physical properties of dairy products (Ye et al 2008). Processing conditions for

homogenization and heat treatment of the formula must be thoroughly tested to ensure nutritional quality, palatability, and stability are not negatively impacted.

3. OBJECTIVES

The objective is twofold: to develop and process a formula for the infant pig that meets nutrient requirements and considers its palatability and stability as well as to assess the digestibility of the protein in the homogenized and heat-treated, high-lipid formula.

4. METHODS

a. Formula Preparation

The formula macronutrient requirement targets were based off a comprehensive review and statistical model of sow milk composition by Hansen et al (2016).

Table 1: Infant pig formula ingredients

Ingredients	g/100mL
WPI 9000	3.00
Micellar casein	2.60
lactose	5.10
PL 700	1.00
vitamin premix	0.05
mineral premix	0.03
butter	8.60

Swine vitamin and mineral premixes (Nutra Blend, Neosho, MO) were pre-dissolved in hot water. Dry ingredients [WPI (Hilmar, CA), micellar casein (Idaho Milk Products, Jerome, ID), lactose (LD Carlson's, Kent, OH), PL 700 (proprietary phospholipid mix)] shown in **Table 1** were premixed and added into hot water in a steam-jacketed 18-gal Breddo Likwifier on the high-speed setting and mixed for 5 minutes; butter (Kroger, Cincinnati, OH) melted at 40°C was incorporated slowly on the high-speed setting. The batch was heated with constant agitation to 60°C and homogenized by a two-stage homogenizer with first and second stage pressures of 500 and 1000 psi. The control was bottled in a positive displacement hood. Remaining formula was processed further with HTST (72°C, 15 sec) or indirect UHT (135°C, 3-5 sec) and then bottled.

b. Proximal Analysis

The moisture and total solid content was quantified on a Smart Trac II instrument (CEM, Mathews, NC), which utilizes Low Resolution Nuclear Magnetic Resonance (LR-NMR), all based on the AOAC Dairy Approved Method – PVM 1:2004 (Cartwright 2005). The lipid content was quantified rapidly on the Oracle (CEM, Matthews, NC) instrument, which also utilizes NMR technology based on the AOAC Dairy Approved Method – PVM 1:2004 by Cartwright (2005). The true protein content was quantified on a Sprint Rapid Protein Analyzer instrument (CEM, Matthews, NC), which is a colorimetric method based on AOAC Method 967.12. The ash content was quantified on a Phoenix microwave ashing system; approximately 2g of sample was ashed for 10 minutes at 800°C. The lactose content was calculated by difference. All analyses were run in triplicate.

c. In-vitro digestion of formulas

The formulas were suspended in 50 mM potassium phosphate buffer at pH 7.5 at a sample to buffer ratio of 1:3. The pH was adjusted with 1 N HCl to a final concentration of 0.04N and pepsin (Promega, Madison, WI) was added at an enzyme to substrate ratio of 1:20. Samples were incubated in a 37°C water bath, agitated by magnetic stirring rods at 80 RPM, and aliquots taken at 3, 5, 10, 15, 20, 30, 60, and 120 min. Enzyme activity was halted by addition of SDS sample buffer and heating at 95°C for 10 minutes in a digital dry bath/block heater (Thermo Fisher Scientific, Waltham, MA). Protein digestibility was evaluated by evaluating the intensity of the of peptide fraction bands in SDS PAGE.

d. Gel Electrophoresis

In-vitro digestion aliquots were dissolved in 2x Laemmli sample buffer (Bio Rad, Hercules, CA) with β-mercaptoethanol at a 1:1 ratio, heated at 95°C for 10 minutes in a digital dry bath/block heater (Thermo Fisher Scientific, Waltham, MA), and subjected to polyacrylamide gradient gel electrophoresis in SDS-PAGE using Stain-Free gels with a gradient of 4-20% (Bio-Rad) in a Mini-

PROTEAN Tetra cell system (Bio Rad) with 1x Laemmli SDS-PAGE running buffer at 180V. Samples were centrifuged at 6000 RPM for 5 minutes, and 10µL of sample was loaded into each well, avoiding the pellet and lipid layer; the Bio Rad Precision Plus standard molecular weight marker was used. Band intensity was quantified using the Bio Rad ChemiDoc Touch imaging system, which is based off activation and quantification of fluorescent Trp residues in protein. Digestion and gel electrophoresis of the samples was done in triplicate.

e. Viscosity

Viscosity of the formulas was measured with a Brookfield RV viscometer model DV-II+ (Stoughton, MA) in triplicate. A #2 spindle at 50 RPM was used to take viscosity readings at 4°C. Viscosity readings taken every 30 seconds for 3 minutes were averaged.

f. Accelerated Sedimentation

Five mL of each sample was centrifuged for 15 min at 4000 RPM in 4°C in triplicate and the results visually examined.

5. RESULTS AND DISCUSSION

As seen in **Figures 1, 2, and 3**, SDS-PAGE patterns of the control, HTST, and UHT-treated formulas resulted in at least four distinct casein bands at ~30kDa, ~28kDa, ~24kDa, ~20kDa and two whey protein bands at ~18 kDa and ~12kDa. Bands at ~30kDa and ~28kDa for α-casein and β-casein were consistent with the Lee and Sherdon (2002) SDS-PAGE pattern of milk proteins in homogenized and heat-treated whole milk.

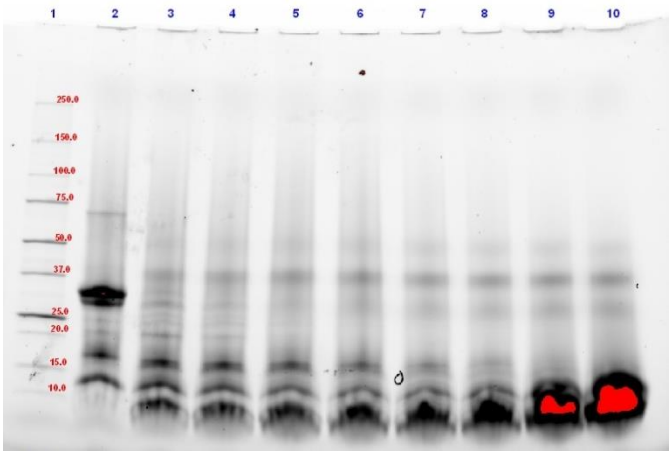

Figure 3: SDS-PAGE gel of control formula: (Lane 1) molecular weight marker, (Lane 2) control without enzyme addition, (Lane 3-10) t=3, 5, 10, 15, 20, 30, 60, 120 min of in vitro digestion

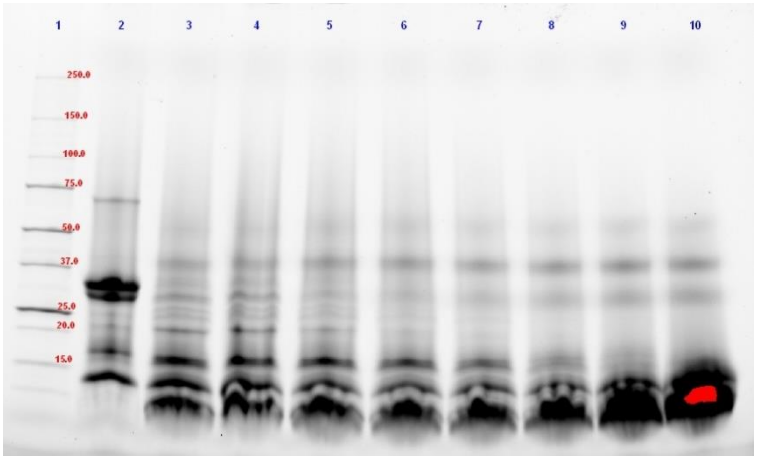

Figure 3: SDS-PAGE of HTST-treated formula: (Lane 1) molecular weight marker, (Lane 2) HTST sample without enzyme addition, (Lane 3-10) t=3, 5, 10, 15, 20, 30, 60, 120 min of in-vitro digestion

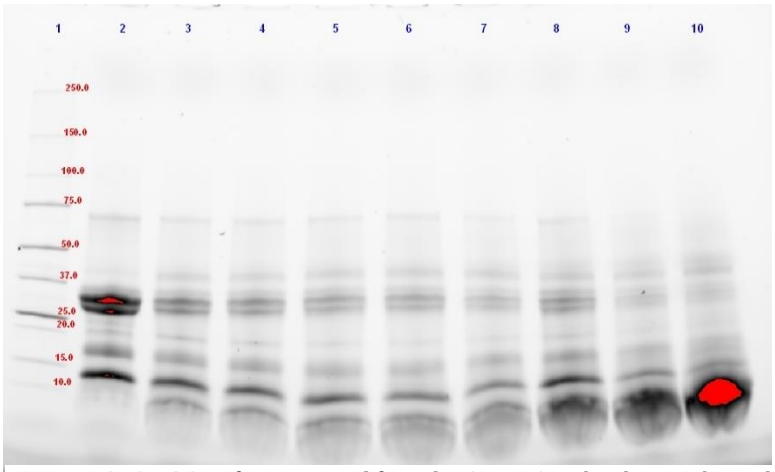

Figure 3: SDS-PAGE of UHT-treated formula: (Lane 1) molecular weight marker, (Lane 2) UHT sample without enzyme addition, (Lane 3-10) t=3, 5, 10, 15, 20, 30, 60, 120 min of in-vitro digestion

Additional noteworthy bands in Lane 1 in **Figures 1-3** can be seen at ~50kDa and ~70kD.The faint bands at ~50kDa that increase in intensity throughout the digestion may be lactadherin, a native MFGM protein that only binds loosely to the membrane (Mather and Keenan 1975). The distinct band at ~70kDa, whose intensity decreases rapidly across the lanes, may be butyrophilin (67 kDa), another native MFGM protein (Lee and Sherdon 2002). Pepsin, with a molecular weight of 34.6 kDa, appears as a consistently faint band in Lanes 2-10.

Figure 4 below shows the general trend for casein bands throughout digestion; their band intensities decrease rapidly in the first 3 minutes and then slowly over the remaining course of digestion, whereas the whey proteins were not broken down as quickly (**Figure 5**); error bars show the standard error of the mean. While the amounts of β-LG in the HTST-treated formula, α-LA in the UHT-treated formula, and the α-LA in the control formula appear to increase in **Figure 5**, the rise in intensity of whey bands towards the end of digestion is likely due to the confounding factor of digested peptides of similar molecular weight.

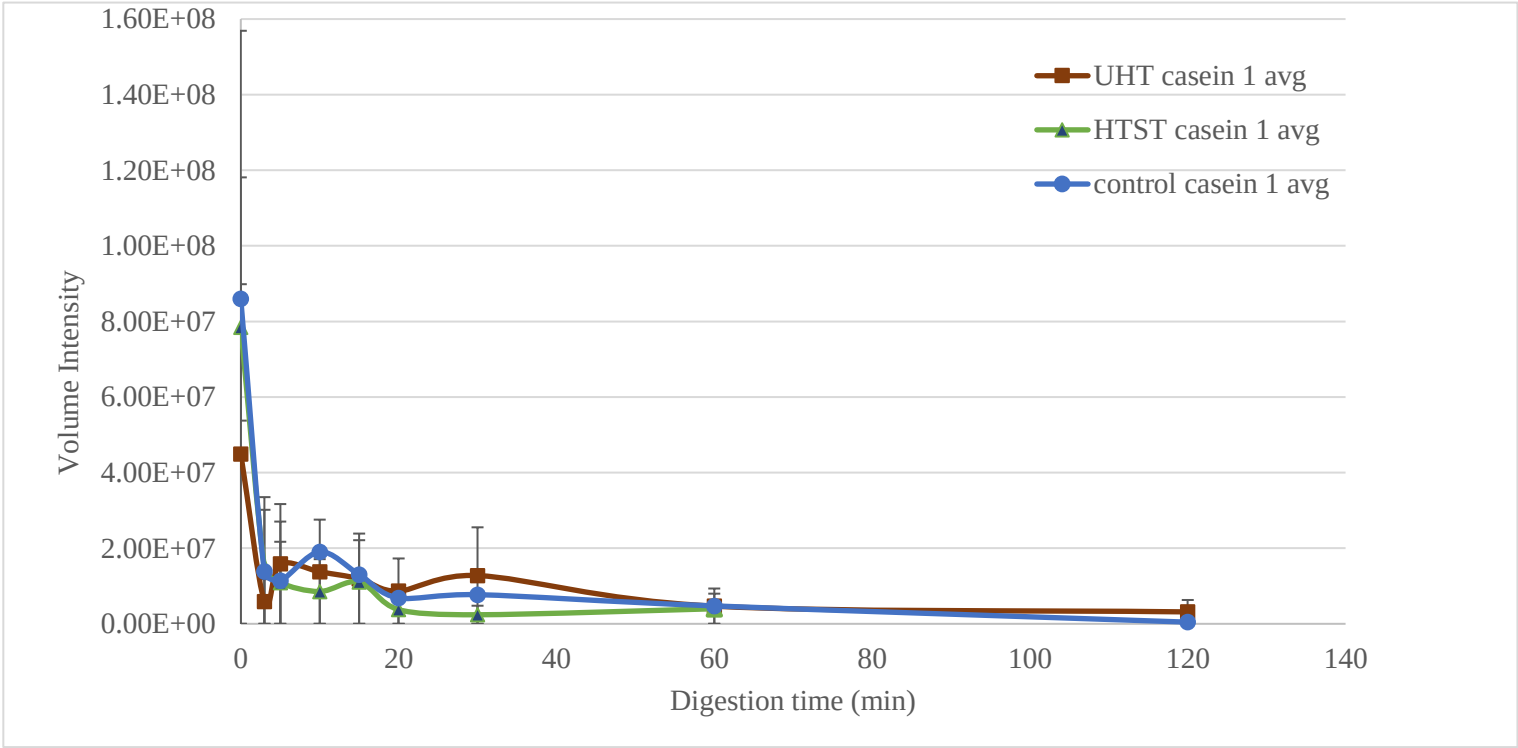

Figure 4 Intensity of α-casein bands throughout in-vitro digestion of control, UHT, and HTST-processed formula

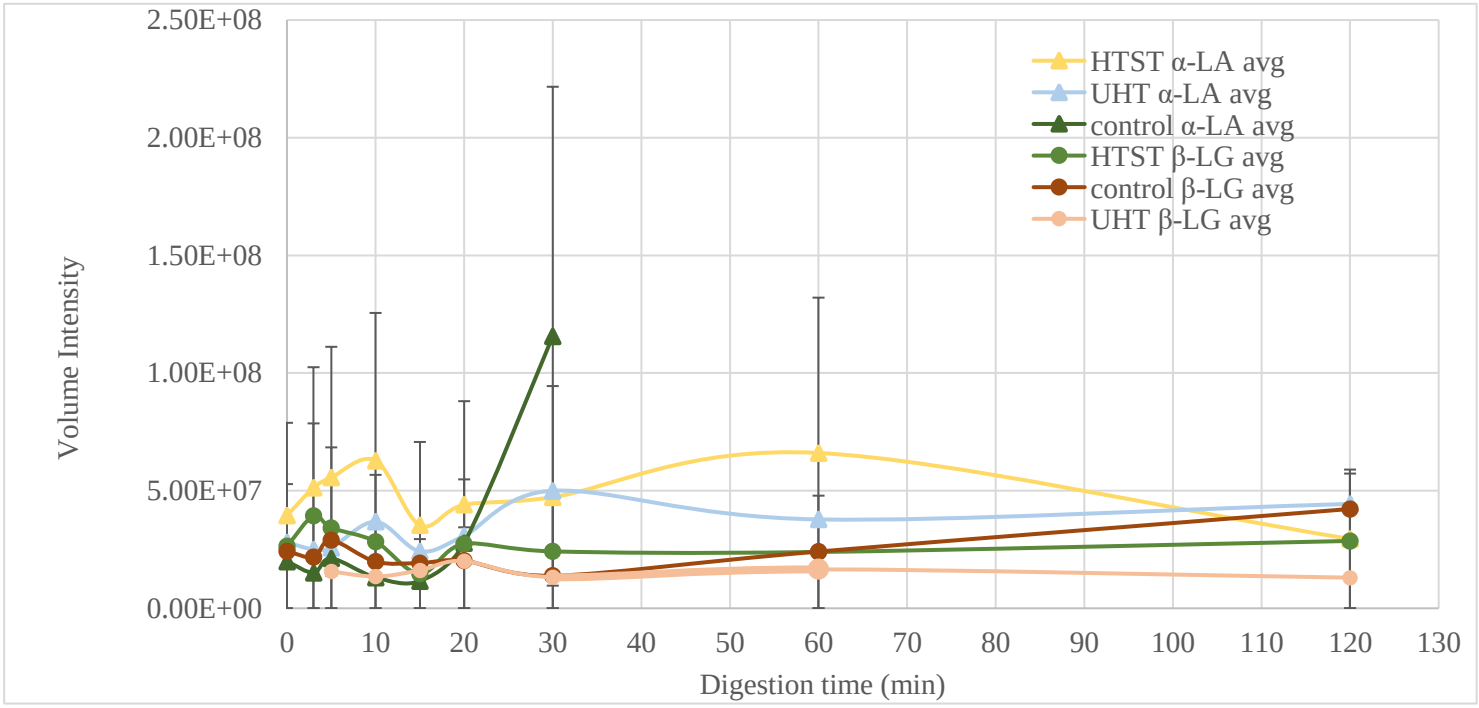

Figure 5 Intensity of α-La and β-LG bands throughout in-vitro digestion of control, UHT, and HTST-processed formula

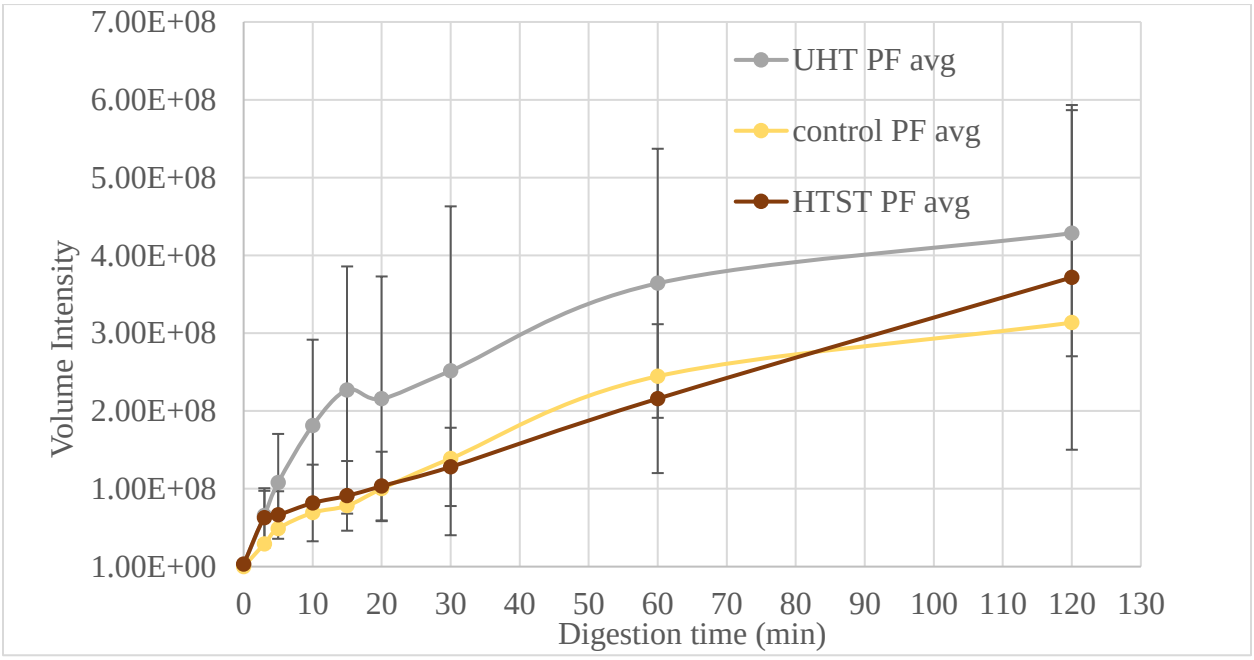

Figure 6 Peptide fraction intensity throughout in-vitro digestion by pepsin of control, HTST, and UHT-processed formula

Figure 6 shows that the UHT formula yielded more intense peptide fraction bands post-pepsin digestion, while the control and HTST formulas exhibited similar protein digestibility. However, the mean standard error of the data is large; conclusions cannot be drawn without more trials. The large variability in the data could be due to pipetting error or the skewing of intensity values due to saturated bands seen in **Figures 1-3.** However, the greatest contributor to error is likely the variability in the processing of the formulas themselves. Inadequate hydration/ reconstitution time led to sedimentation of solids in the formula, resulting in a non-homogenous mixture and variable solids content among bottles. **Table 3** below shows non-homogeneity of lipid, protein, and lactose content among the control, HTST, and UHT samples. Variable protein concentrations among samples would lead to great variability in the intensities of peptide fractions.

Table 3: Proximal analysis of control, HTST, and UHT-processed formula

Component (g/100mL)	Control			HTST			UHT			Target
moisture	83.53	±	0.17692	84.31	±	0.5656	83.5767	±	1.0451	80.83
total solids	16.47	±	0.17692	15.69	±	0.5656	16.4233	±	1.0451	19.17
lipid	6.54	±	0.10	5.75	±	0.46	6.44	±	0.56	8.00
protein	6.02	±	1.65	8.29	±	0.98	6.68	±	1.76	5.10
lactose	3.49	±	1.86	1.04	±	1.23	2.83	±	2.29	5.39
ash	0.37	±	0.10	0.53	±	0.07	0.41	±	0.12	0.68

Inadequate hydration has multiple implications. It results in sedimentation of solids, thus decreasing the macronutrient content of the formula and resulting in suboptimal nutrient intake by the baby pigs. Additionally, inadequate hydration may lead to severe processing difficulties. Non-hydrated particulates will put strain on homogenizers, which work by forcing the milk fat

globule through a small opening and can easily lead to fouling of heat exchangers during heat-processing. Fouling would only worsen with continued processing, eventually leading to clogging of the heat-exchanger, which could then cause overheating and coagulation of casein proteins if temperatures reach 120-140°C (Singh 2004). It is our hypothesis that this occurred, as only a few liters of UHT product were processed through the tube-in-tube heat exchanger before pressure increased in the pipes and the UHT system became completely clogged by coagulated casein protein (see appendix). In the accelerated sedimentation study, all samples developed a white pellet of solids during centrifugation (see appendix). However, only the UHT sample had a non-opaque supernatant, which indicated that the casein micelles might have been disrupted by the high heat and that the formula might have lost its colloidal suspension.

Table 4: Viscosity of control, HTST, and UHT-treated piglet formulation

	viscosity (cP)		
control	4.789	±	0.463
HTST	4.900	±	0.283
UHT	10.811	±	0.277

The UHT processing also increased the viscosity of the infant pig formula, as shown in **Table 4.** Viscosity of the samples was taken to ensure similarity of viscosity of the formulated samples to sow's milk, which is approximately 3.850 cP (Whittlestone 1952). The data suggests that homogenization and UHT treatment increases the viscosity of the formulation. These results were consistent with Lee and Sherbon's study in which the viscosity of whole milk increased significantly after homogenization and heat treatment (2002). While UHT sterilized piglet formula may provide many advantages, the palatability of a formula more viscous than sows milk, a potential "cooked" flavor due to sulphur and carbonyl flavor compounds (Zabbia et al 2012) are important considerations.

6. CONCLUSION

Though these results on whether homogenization and heat processing affects protein digestibility of sow's milk replacement are inconclusive, the findings here are key for future development. Complete hydration of the formula before processing is paramount for successful and consistent subsequent homogenization and heat treatment. It is recommended that the ingredients of the formula be hydrated in the Breddo at low-speed for at least two hours or until rapid sedimentation no longer occurs. Moreover, the viscosity of the formula should be reduced to better mimic the viscosity of sow's milk. One might also consider addition of a commercial flavor proven to be favorable to piglets to address the potential cooked flavor of UHT samples. It is evident that in the development and processing of sow's milk replacement for artificial rearing of pigs, there are many considerations dealing with nutrient composition, digestibility, palatability, and stability; processing conditions must be chosen carefully and validated. Further trials should be performed on the viability of HTST and UHT processing of 8% (w/v) lipid piglet formula and the effects of this processing on the MFGM and resulting protein digestibility.

7. REFERENCES

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